Hyperexpression and Analysis of choB Encoding Cholesterol Oxidase of Brevibacterium sterolicum in Escherichia coli and Streptomyces lividans.

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We examined the expression of choB, encoding cholesterol oxidase of Brevibacterium sterolicum ATCC 21387, in Escherichia coli JM1105 and Streptomyces lividans TK23 using various deletion DNA fragments within the 5'-flanking region. The enzyme activity could be detected intracellularly in E. coli only when the 5'-flanking region was reduced to less than 256-bp and choB was transcribed by the lac promoter. A large amount of the enzyme were produced as inactive inclusion bodies when ChoB protein was fused with the NH2-terminal portion of LacZ protein. In contrast, choB with more than 256-bp of the 5'-flanking region was efficiently expressed in S. lividans TK23, and about 85 times as much of the active enzyme (170 U/ml) was secreted into the culture filtrate as with B. sterolicum in flask culture. These results suggest that the promoter of choB exist within 256-bp of the 5'-flanking region and can be efficiently recognized by the RNA polymerase of S. lividans. The characteristics of the enzyme purified from the culture filtrate of the S. lividans transformant and that of B. sterolicum were identical although the NH2-terminal amino acid sequence of the enzyme from the S. lividans transformant was 6 amino acids shorter than that from B. sterolicum.

Cholesterol oxidase (EC 1.1.3.6) catalyzes the oxidation of cholesterol (5-cholesten-3β-ol) to 4-cholesten-3-one with reduction of oxygen to hydrogen peroxide, and is one of the key enzymes in cholesterol metabolism. This is used for the measurement of total serum cholesterol coupled with cholesterol esterase and peroxidase.31 Cholesterol oxidases are produced by Brevibacterium,32 Rhodococcus,33 Streptomyces,4,57 Schyzophyllum,69 and Streptoverticillium71 species. Brevibacterium sterolicum ATCC 21387 produces cholesterol oxidase in the culture filtrate.27 We previously cloned the gene (choB)90 and analyzed the nucleotide (nt) sequence.90 The gene encodes a 507 amino acid (aa) mature protein of the predicted M, 54902 preceded by a 45 aa signal sequence. Comparison of the nt and aa sequences to those of the cholesterol oxidase gene (choA) of Streptomyces sp. SA-COO10 showed significant similarity of 64% and 58%, respectively.

Cloned genes containing choB with more than a 2.5-kb upstream region were not expressed in E. coli.88 One purpose of this study was to improve the yield of the cholesterol oxidase by recombinant DNA techniques. In this study, we examined the overexpression of choB in E. coli and S. lividans host-vector systems, and succeeded in producing the active enzyme in large amounts by S. lividans. We also analyzed the control region of choB for expression, using various deletion DNA fragments within the 5'-flanking region.

Materials and Methods

Bacterial strains and DNA. Escherichia coli JM105 and Streptomycetes lividans TK23 were used as the hosts for the gene manipulation and the expression of choB. Plasmids pUC1911 and pJ70212 were used as vectors for E. coli and for S. lividans, respectively. Shuttle plasmids between E. coli and S. lividans, pSE1 and pSE2 (Fig. 1), were constructed from pUC19 and pJ702 using their PstI sites. The 5.7-kb EcoRI–HindIII fragments of pSE1 and pSE2 include the intact pJ702, and they were used as the vectors to introduce choB into S. lividans. Construction of pSca8 containing choB was previously described.89

Generation of the deletion fragments. A 2.4-kb MluI fragment of pSca8, which contained choB with 616-bp of the 5'-flanking region, was blunt-ended by T4 DNA polymerase and inserted into the Smal site of pUC119 (Fig. 1). The resultant plasmid, pCHS43, contained choB in the same direction as the lac promoter. A plasmid pCHS43-445 was obtained from pCHS43 by digestion with SphI and re-ligation. To obtain the various deletions to different extents within the 5'-flanking region and/or the signal sequence of choB, pCHS43 was digested with PstI and blunt-ended with T4 DNA polymerase, digested with Spel and deleted unidirectionally from the PstI site using a Kolo-sequencing Deletion Kit (Takara Shuzo).12 These DNA preparations were ligated and introduced into E. coli JM105. The structure of each plasmid was confirmed by DNA sequencing using a M13 reverse primer (Takara Shuzo).

Construction of plasmids for the expression of choB in S. lividans. The 2.4-kb MluI fragment of pSca8 was inserted between the two MluI sites in mel of pJ702 (Fig. 1), and S. lividans TK23 was transformed by this DNA preparation by the method of Chater and Hopwood.14 The structural gene of choB was inserted in the same and opposite directions to the mel promoter in the resultant plasmids pCHS31 and pCHS32, respectively. The EcoRI–HindIII fragments of the plasmids containing the various deletion fragments generated from pCHS43 mentioned above were ligated to 5.7-kb EcoRI–HindIII fragments of pSE1 and pSE2. S. lividans TK23 was transformed by these DNA preparations.

Media and cultivation conditions. E. coli transformants were grown in 60 ml of LB or L2G (LB plus 2% (w/v) glucose) medium containing 25 μg/ml ampicillin at 37°C for 4 h. When the lac promoter should be induced, 10 μg/ml of isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to LB medium at the inoculation time. S. lividans transformants were grown at 30°C in SK No. 2 medium (20% (w/v) Stabilose K (Matsutani Kagaku), 0.5% (w/v) glucose, 0.5% (w/v) yeast extract (Nippon Seiyaku), 0.5% (w/v) peptone (Nippon Seiyaku), 0.3% (w/v) meat extract (Kyokuto Seiyaku), 0.02% (w/v)
Fig. 1. Construction Strategies of the Plasmids.
KH₂PO₄, 0.06% (w/v) MgSO₄·7H₂O, pH 7.6) at 30°C. Thiopeptin (Fujisawa Pharmaceutical) was added at 20 μg/ml when the strain carried one of the pJ702-derived plasmids.

Assay of the enzyme activity. Cells of *E. coli* and *S. lividans* transformants were collected by centrifugation at 8000 × g for 20 min at 4°C, and the extracellular activities in the supernatants were measured. The cells were washed with 20 mM sodium potassium phosphate buffer (pH 7.5) twice, and disrupted by sonication using a Tomy Seiko Model UR-200 sonicator. The sonicated preparations were centrifuged at 20,000 × g for 15 min and the supernatants were assayed for the activities of the intracellular fractions. The pellets were designated as the intracellular insoluble fractions. The assay conditions of the enzyme activities were previously described.9)

Purification of the extracellular cholesterol oxidase from *S. lividans* TK23(pCHS31). *S. lividans* TK23(pCHS31) was grown at 30°C in 150 h in SK No. 2 medium containing 20 μg/ml of thiopeptin. The culture supernatant was obtained by centrifugation at 8000 × g for 20 min at 4°C, and directly separated by isoelectric focusing in 2% (v/v) Bio-lyte 3/10 using a Rotofor preparative isoelectric focusing cell (Bio-Rad). The fraction including the cholesterol oxidase was selected by the assay of the enzyme activity and the SDS–PAGE analysis. Bio-lyte was eliminated by ammonium sulfate precipitation, the pellet was dissolved in 10 ml of 20 mM sodium–potassium phosphate buffer (pH 7.5), and the preparation was subsequently dialyzed for 16 h at 4°C against 200 volumes of the same buffer. Enzyme activity and optical absorbance at 280 and 470 nm of the preparation were measured. The protein concentration was calculated from the molecular extinction coefficient (ε1% at 280 nm = 17.1) of the purified cholesterol oxidase from *B. stercorium*.15)

Analysis of the NH₂-terminal amino acid sequence. The enzyme was purified from the culture supernatant of *S. lividans* TK23(pCHS31) as described above, and desalted by reverse-phase high performance liquid chromatography using Brownlee C4 column (2.1 × 30 mm). The purified enzyme was sequenced using an Applied Biosystems Model 470A Gas Phase Sequencer. The resultant PTH-amino acids were identified using an Applied Biosystems Model 120A PTH Analyzer with Brownlee PTH-C18 column (2.1 × 220 mm).

Results

Expression of choB by the lac promoter in *E. coli*

A DNA fragment containing choB with more than 2.5-kb of the 5′-flanking region was not expressed in *E. coli*.30) DNA sequence analysis confirmed that the 2.4-kb *MluI* fragment of pSc8a contained the entire structural gene of *choB* together with the upstream 616-bp of the 5′-flanking sequence.9) Then, we examined the possibility of the expression of *choB* by external transcription readthrough from the lac promoter. A plasmid pCHS43 was constructed in which the *MluI* fragment was cloned into the Smal site of pUC119 to locate *choB* in the same direction as the lac promoter. However, no enzyme activity was detected in *E. coli* JM105(pCHS43). This suggests existence of a structure which prevents the transcription readthrough in the 616-bp of the 5′-flanking region. Thus, deletion plasmids were constructed from pCHS43 to remove the 5′-flanking sequence to different extents (Fig. 2).

Table 1 shows the intracellular enzyme activities of the *E. coli* JM105 transformants harboring these plasmids. High activities were detected in *E. coli* JM105(pCHS43−24) and JM105(pCHS43−25), in which only 24 and 25-bp upstream from the initiation codon remained, respectively. *E. coli* JM105(pCHS43−82), in which *choB* lacked a part (82-bp) of the signal sequence and was fused to the NH₂-terminal portion of β-galactosidase, also showed comparable activity. These activities were enhanced in the presence of IPTG and repressed by glucose. On the other hand, *E. coli* JM105(pCHS43−255) containing *choB* with 255-bp of the 5′-flanking region showed only a little activity. These results indicated that the inhibitory sequence for the transcription from the lac promoter existed in −255 to −25 in the 5′-flanking region. No activity was detected in either *E. coli* JM105(pCHS43+2) or JM105(pCHS43+110) which lacked the initiation codon.

In *E. coli* JM105(pCHS43−24) and *E. coli* JM105− (pCHS43+82), inclusion bodies were observed in the cells. Significant amounts of the ChoB protein were found in the intracellular insoluble fractions by SDS–PAGE (Fig. 3). The molecular weights were larger than that of the enzyme purified from the culture filtrate of *B. stercorium*. They were coincident with those of the predicted fusion proteins, β-galactosidase-ChoB with the *choB* signal sequence; 61 kDa in pCHS43−24 and 54 kDa in pCHS43+82, respectively. Cholesterol oxidase of *B. stercorium* is a
Expression of the Cholesterol Oxidase Gene

Table 1. Expression of choB by the lac Promoter in E. coli

<table>
<thead>
<tr>
<th>Strains</th>
<th>Intracellular enzyme activity (mU/mg protein)</th>
<th>Extracellular enzyme activity (U/ml broth)</th>
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<tr>
<td></td>
<td>LB</td>
<td>IPTG(−)</td>
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<td>E. coli</td>
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<td>JM105 (pUC119)</td>
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<td>176</td>
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<tr>
<td>JM105 (pCHS43+82)</td>
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<td>357</td>
</tr>
<tr>
<td>JM105 (pCHS43+110)</td>
<td>ND *</td>
<td>ND</td>
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* Numerals after + or − in the plasmid names represent the nucleotide positions of the termini from the choB initiation codon in the deleted DNA fragments.
+ . ND represents “not detected”.

**Fig. 3. Analysis of the Products of E. coli JM105(pCHS43−24) and E. coli JM105(pCHS43+82) by SDS-PAGE.**

SDS-PAGE patterns of the total cellular proteins, intracellular proteins, and extracellular insoluble proteins of E. coli JM105(pCHS43−24) and E. coli JM105(pCHS43+82) are indicated. Lane 1, size marker (94, 67, 43, 30, 20, and 14.4 kDa); lane 2, 0.025 units of cholesterol oxidase purified from the culture filtrate of B. stearothermophilus; lanes 3−5 are preparations from E. coli JM105(pCHS43−24); lanes 6−8 are preparations from E. coli JM105(pCHS43+82); lanes 3 and 6 are intracellular total proteins; lanes 4 and 7 are intracellular proteins; and lanes 5 and 8 are intracellular insoluble proteins. Arrows indicate the size of 55, 57, and 61 kDa.

flavoenzyme. However, the inclusion bodies seemed not to contain FAD molecules judging from the lack of typical fluorescence originated from the prosthetic group. A proper supply of the cofactors might be one of the important factors in the synthesis of cofactor-containing enzymes. The optimal conditions for dissolving and renaturation of the inclusion bodies and for introducing FAD molecules to obtain a fully active enzyme need to be discovered.

Expression of choB in S. lividans

It is known that heterologous genes from various prokaryotes can be expressed in S. lividans.16,17 We examined the expression of choB in S. lividans TK23. First, the 2.4-kb MluI fragment of pSCa8 containing choB with 616-bp of the 5′-flanking region was introduced between the two MluI sites within mel of pIJ702. The choB gene was inserted in the same and the opposite directions to the mel promoter in pCHS31 and pCHS32, respectively (Fig. 1). Both S. lividans TK23(pCHS31) and TK23(pCHS32) secreted significant amounts of the enzyme into their culture filtrates (Table II). The productivity of S. lividans TK23(pCHS31) was a little higher than that of S. lividans TK23(pCHS32).

Next, we examined the expression of the deletion fragments described above to identify the locus which promoted the expression of choB in S. lividans TK23. The EcoRI−HindIII fragments of pCHS43, pCHS43−445, pCHS43−25, pCHS43−24, pCHS43+2, pCHS43+82, and pCHS43+110 were ligated to the EcoRI−HindIII fragments of pSE1 and pSE2 containing entire pIJ702. Plasmids pCHS51, pCHS51−445, pCHS51−25, pCHS51−24, pCHS51+2, and pCHS51+82 were derived from pSE1, and choB was inserted at the position of PstI of pIJ702 in the same direction as mel. Plasmids pCHS52, pCHS52−445, pCHS52−25, pCHS52−24, pCHS52+2, and pCHS52+82 were derived from pSE2, and choB was inserted in the opposite direction. S. lividans TK23(pCHS51), TK23(pCHS52), TK23(pCHS51−445), TK23(pCHS52−445), TK23(pCHS51−25), and TK23(pCHS52−25), which include more than 256-bp upstream from choB in both directions, secreted the enzyme at almost the same level as S. lividans TK23(pCHS31) did. These results indicated that the choB expression in S. lividans TK23 was promoted by the sequence within the 5′-flanking 256-bp of choB.

Production of cholesterol oxidase by S. lividans harboring pCHS31

We chose S. lividans TK23(pCHS31) for detailed analysis of the production of the recombinant cholesterol oxidase. The strain was grown aerobically at 30°C in a 2-liter flask containing 500 ml of SK No. 2 medium supplemented with 20 µg/ml thiopentin. The activity of the extracellular enzyme was significantly increased after 90 h of cultivation and reached more than 170 U/ml at 120 h (Fig. 4A). The enzyme was efficiently secreted and more than 85% of the total activity was detected in the culture filtrate. The enzyme accumulated in the culture filtrate was about 85 times higher than that by the original B.
points, were identical with each other (data not shown).

Discussion

The reason why the DNA fragment containing choB with more than 2.5-kb of the 5'-flanking region was not expressed in E. coli could be explained in two ways, either the promoter sequence does not exist in this region, or cannot be recognized by RNA polymerase of E. coli. In the case of Streptomyces sp. strain SA-COO, another producer of cholesterol oxidase, the promoter does not exist just upstream from the structural gene but appears more than 1.2-kb upstream, and the gene (choA) is transcribed polycistronically with another ORF encoding a P450-like protein preceding choA.\(^1\)\(^8\) We found a potential ORF, or a portion of it, upstream from choB, but no homology to the P450-like protein could be observed while significant homology was observed between choB and choA structure genes.\(^9\) The potential ORF may not organize an operon with choB because the transcription readthrough from the lac promoter was prevented at the upstream region of choB in E. coli JM105(pCHS43). This suggests that the gene organizations and regulations would be different between choB and choA from these two bacteria.

The experiment promoting the expression by the external lac promoter in E. coli showed that a structure which prevented the readthrough existed with the 255-bp region upstream from the choB initiation codon. A small palindromic sequence (capable of forming a stem-and-loop structure in RNA with ΔG = −13.8 kcal/mol, which was calculated according to the rules of Tinoco\(^1\(^9\)\) was found 186–165-bp upstream from the initiation codon. Although the value of ΔG is smaller than that observed in the 3'-flanking region of choB, which might act as the transcription terminator, the sequence might reduce the readthrough from the lac promoter. This sequence was found within the predicted ORF preceding to choB. The physiological role of this sequence in B. sterolicum is unclear, but a negative control element, such as an attenuator, might exist in this region.

Hyperexpression of choB was observed in both S. lividans TK23(pCHS51) and TK23(pCHS52), in which the MluI fragment was introduced at the position of the PspI site of pIJ702 in both directions. External transcription readthrough could be negligible at this position because only traces of activity were detected in either S. lividans TK23(pCHS51 – 25) or TK23(pCHS52 – 25) containing the intact choB with its ribosomal binding sequence. This indicated that the expression in S. lividans TK23(pCHS51) and S. lividans TK23(pCHS52) was promoted by the sequence within the 255-bp of the 5'-flanking region. In contrast, the same DNA fragment was not expressed in E. coli JM105(pCHS43 – 255). These results suggested that the promoter choB could be recognized by RNA polymerase in S. lividans TK23 but not by that in E. coli JM105.

No sequence homologous to the E. coli promoter was observed in the upstream region of choB.\(^9\) Moreover, no sequence matched to the promoter sequences defined in Streptomyces\(^2\)\(^0\) could be found in this region. It is known that there is a multiplicity of the sigma factors of RNA polymerase in Streptomyces,\(^2\)\(^1\) and there should be several sequences which act as functional promoters cor-

Fig. 4. Production of the Recombinant Cholesterol Oxidase by S. lividans TK23(pCHS31).

(A) Enzyme activity of S. lividans TK23(pCHS31). X axis represents the cultivation time (h). Enzyme activities in the culture filtrates and the intracellular fractions are represented by a plane line with open boxes and a dashed line with closed circles, respectively. A dotted line with open circles represent packed cell volume (PCV, ml cells per ml broth). (B) Analysis of the products of S. lividans TK23(pCHS31) by SDS-PAGE. Lanes 1–6 are the culture filtrates from 12 μl of culture broth, and lanes 7–12 are the intracellular fraction from 12 μl of culture broth. Lane 7 has the size markers (phosphorylase b = 94 kDa, 128 μg/lane; bovine serum albumin = 67 kDa, 166 μg/lane; ovalbumin = 45 kDa, 294 μg/lane; carbonic anhydrase = 30 kDa, 1.66 μg/lane; soybean trypsin inhibitor = 20 kDa, 1.60 μg/lane; α-lactalbumin = 14.4 kDa, 2.42 μg/lane). Lanes, 1 and 8 are at 27.5 h; 2 and 9 are at 46.0 h; 3 and 10 are at 70.0 h; 4 and 11 are at 99.0 h; 5 and 1 are at 128.0 h, and 6 is at 151.0 h. Arrow represents the position of the size of the extracellular recombinant enzyme.
responding to these sigma factors. The promoter of choB might be responding to one of the recognition sequences of these sigma factors of S. lividans TK23.

S. lividans TK23(pCHS31) secreted more than 170 U/ml of the recombinant cholesterol oxidase in 2-liter flask culture, corresponding to about 0.6 g/liter calculated from the specific activity of the purified enzyme. The productivity will be improved by further investigation of the cultivation conditions. The specific activity of the purified recombinant enzyme was 287.7 U/mg protein. In the SDS-PAGE analysis of the culture filtrate 2.4 U enzyme was estimated to be approximately 8.5 μg from the density of the corresponding band [Fig. 4(B) lane 4]. This suggests that the recombinant cholesterol oxidase produced by S. lividans TK23(pCHS31) is fully active. They could contain almost 1 mol of FAD per 1 mol of protein judged from the ratio of F280nm/F470nm of the purified recombinant enzyme. These results indicate that the supplement of the FAD molecule is not limited in the production of the active recombinant cholesterol oxidase at this level in S. lividans TK23, unlike E. coli.

The extracellular enzyme produced by S. lividans TK23-(pCHS31) lacked 6 aa in the NH₂-terminus compared to that of B. sterolicum. The sequence preceding the NH₂-terminus of the product from S. lividans TK23(pCHS31) seemed to be different from typical sequences around the signal processing sites. A typical sequence, Ala-Xaa-Ala, was observed adjacent to the NH₂-terminus of the extracellular enzyme from B. sterolicum. Consequently, the lack of the 6 aa of the NH₂-terminus in the recombinant enzyme might be caused by processing after the secretion by an extracellular protease of S. lividans TK23. However, the characteristics of the recombinant enzyme such as the thermostability showed good agreement with those of the original enzyme of B. sterolicum (data not shown).

The recombinant cholesterol oxidase produced by the S. lividans TK23 transformants were quite stable in the culture filtrates. The skills of the cultivation on an industrial scale are well known for Streptomyces species because they have been used as the producers of a lot of industrial materials such as enzymes and antibiotics for a long time. We believe that S. lividans is a good host for industrial productions of the recombinant cholesterol oxidase.

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References